THE GERM-CELLS AND THE PROCESS OF FERTILIZATION IN THE CRAB, MENIPPE MERCENARIA

BY

RAYMOND BINFORD

A DISSERTATION

Submitted to the Board of University Studies of the Johns Hopkins University in conformity with the Requirements for the Degree of Doctor of Philosophy.

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NINE PLATES

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[1] The study of the life-history of Menippe mercenaria, which led to the discoveries presented in this paper, was undertaken at the suggestion of Prof. E.

1. INTRODUCTION

In spite of the extensive researches into the spermatogenesis of the Decapods, the use of the peculiar structures found in the spermatozoa of these animals is still an unsolved problem. This is due to the fact that the entrance of the spermatozoon into the egg has never been reported. While studying the habits and structure of Menippe mercenaria, a large edible crab found along the southern part of the Atlantic coast of the United States, I had the good fortune to obtain material which shows the essential features of this process. In order to show which parts of the seminal cell are involved in the process of fertilization, the genesis of the spermatozoa and the formation of the pronucleus in the fertilization of the egg, as well as the entrance of the spermatozoon, are here described. The history of the male cell from its origin in the epithelium of the wall of a tubule of the testis to its association with the female nucleus in the center of the egg, is here presented.

2. SPERMATOGENESIS

The testis

The testis of Menippe is a large paired organ lying just underneath the dorsal wall of the carapace. The inner ends of the right and left portions lie close together, just anterior to the heart and from here diverge anteriorly and laterally to the outer edge of the carapace. It is composed of relatively long and complexly folded tubules which vary in diameter from 0.14 to 0.33 mm. The deferent duct, one on each side, leads from the

A. Andrews, and at every step in the progress of this work I have received his kind advice and helpful criticism. I am also greatly indebted to the Hon. Geo. M. Bowers, United States Commissioner of Fish and Fisheries, for the privilege of working in the Marine Biological Laboratory at Beaufort, North Carolina, and for the liberal help extended to me in carrying on my researches there. My thanks are also due to Mr. H. D. Aller, Director of the Laboratory, for his ready cooperation in placing at my disposal the conveniences necessary for carrying forward my work.

testis to the base of the last thoracic leg. It is extensively convoluted so as to form two large masses, one lateral to the posterior part of the testis and the other beneath the posterior part of the heart. The deferent duct is lined with a layer of columnar epithelium which secretes the substance that forms the walls of the spermatophores.

Methods

Pieces of the testis, obtained by cutting across the organ, were fixed in Worcester's fluid. This is a saturated solution of sublimate in 10 per cent formalin. Other fixing fluids were used but did not give as satisfactory results. The sections were cut 7 μ to 10 μ thick. The stains used were thionin and eosin, safranin and Lichtgrün, iron-hematoxylin, and Delafield's hematoxylin.

The testicular tubules

The walls of the tubules of the testis are thin and contain flattened nuclei. Figures 1, 2, 3 and 4 are drawings of transverse sections of the tubules and show different stages in the development of the seminal elements. In figure 4 the tubule is seen to be made up of three regions: The smallest one which borders the more sharply curved side at the bottom of the drawing contains mature spermatozoa. Next to this, and filling the central region, is a space filled with spermatozoa nearly mature. The third region, which forms the crescent shaped portion on the upper side, contains spermatocytes in the early prophase of the first maturation division. There are more or less definite layers of epithelial cells between the different regions of the tubule. The outer wall and sometimes these inner partitions which border the regions containing mature spermatozoa, become thick and columnar in structure (figs. 3 and 4).

Not only do the seminal elements in these separate parallel cavities of the tubule differ in the stages of their development but in the same cavity the elements at one end of the tubule

are further along in their development than those at the other end. Thus in one end of a cavity the cells may be in the early prophase of the division of the spermatocytes of the first order while in the other end they have reached the spermatid stage. All the stages in the transformation of a spermatid into a spermatozoon may be found in passing from one end of a tubule to the other.

At the center of the upper border of figure 4, *p.s.*, there is one cell with a large nucleus. This is one of a single row of cells along the side of the tubule which may be called the primary spermatogonial cells since they, by division, give rise to a new lot of spermatogonia. Near the top of figure 3, *p.s.*, we find a similar cell. The cells forming the crescent-shaped region are in this case not so far advanced as in figure 4. Figure 1 represents a tubule, the largest portion of which is filled with spermatids which have already entered upon their transformation into spermatozoa. In the upper portion of the drawing we have an early stage in the formation of a new batch of spermatogonia. There are four large spermatogonial nuclei surrounded by many epithelial nuclei and a considerable amount of cytoplasm. Delicate cell walls cutting out the cytoplasm which belongs to each spermatogonial cell can sometimes be made out at this stage. A later stage in the multiplication of these cells is shown in figure 2. A large nucleus, *p.s.*, near the middle of the convex border of the spermatogonial mass, doubtless marks the position of the row of primary spermatogonial cells which will persist unmodified to form, at a later period, another lot of spermatogonia. The largest cavity of this tubule contains spermatids well advanced in their transformation into spermatozoa. In figure 3 the mass of spermatogonial nuclei is still further enlarged. Indeed most of them have probably reached the spermatocyte stage. The spermatocytes in the early prophase of the first maturation division are shown in figure 4. By putting these observations together we may determine the approximate order of events in the genesis of the spermatozoa.

A general statement of spermatogenesis

There persists along one side of the tubule a single row of cells with large nuclei, the division of which give rise to the spermatogonia. The latter multiply irregularly to form a large mass which, in transverse sections, has the shape of a crescent. At first cell walls can be made out, but later the nuclei seem to lie in an undivided mass of cytoplasm. Gradually the division of these nuclei ceases and a spireme is formed within each of them. The division up to this time has taken place without the formation of any spireme structure. The appearance of the latter is the first indication that the cells have reached the spermatocyte stage. After the spireme has been formed the nuclei pass into synapsis which lasts for a comparatively long time, so that all the nuclei in a considerable portion of a tubule will be found in this stage at the same time. After synapsis, cell walls are formed in the cytoplasm which persist up to the anaphase of the first maturation division. In the nucleus the chromosomes are formed and the maturation divisions follow one another in quick succession. They begin at one end of the tubule and pass along it like a wave, so that the spindle-figures are found in only a small section of the tubule at any given time. While these events are taking place within the tubule the cells in the wall of the latter multiply so that the wall becomes considerably thickened. The primary spermatogonial cells also divide to start a new group of spermatogonia. Between these cells and the spermatocytes there is always a layer of epithelial cells which persist to form the partitions between the two successive batches of seminal elements. The mass of spermatogonial nuclei remains small until the spermatids are well advanced in their transformation into spermatozoa.

As the mass of spermatogonia increases, the developing spermatozoa are crowded more and more to one side of the tubule. These spermatozoa reach their mature state before the second batch enter synapsis. The epithelial cells surrounding the mature spermatozoa, we may suppose, secrete a fluid which, together with the increasing mass of spermatogonia, press the mature

spermatozoa out of the tubule. This process is not completed, however, before a third batch is formed or even a fourth started.

Spermatogonial mitoses

In the resting spermatogonial nucleus the chromatin is arranged in a loose net-work with enlargements at various places (fig. 5). This net, for the most part, lies just inside the nuclear membrane, the central part of the nucleus containing almost no staining material. The behavior of the chromatin during the prophase of mitosis is as follows: the knots of chromatin become enlarged and more regular in outline while the connecting threads become smaller and disappear. The chromatin finally assumes the form of a large number of paired spheres (fig. 6). An effort was made to count these spheres and numbers were obtained as follows: 51, 55, 57, 58, 62, 62, 68 and 80. One may not, however, place very much dependence in these numbers for some of the spheres are always somewhat aggregated in one or two places so that they can not be definitely distinguished. These chromosomes at first lie in the outer part of the nucleus just inside the nuclear membrane, but are later massed in the center, from which condition they move to their positions in the equatorial plate. Figure 7 is an optical section of the nucleus showing the peripheral arrangement of the chromosomes. In the metaphase and anaphase of the mitosis, the members of each pair are separated from each other and pass to opposite poles of the spindle (fig. 8). These divisions of the spermatogonial nuclei do not occur simultaneously throughout the mass, but singly here and there among the nuclei. The spermatogonia become smaller as they become more numerous.

Maturation mitoses

Finally the spermatogonial divisions cease and the nuclei prepare for the reduction divisions. The quantity of chromatin seems to increase and the spireme makes its appearance. At first it is very long and slender and complexly folded all through the nucleus. The iron-hematoxylin stain can be controlled so

that the spireme has the appearance of a brown thread with granules distributed irregularly along it (fig. 9). The diameter of the granules is slightly greater than that of the thread between the granules. The spireme now becomes shorter and thicker and is finally massed at one side of the nucleus in the condition of synapsis (fig. 10). This stage persists for a comparatively long period. The spermatocytes enter synapsis irregularly, in a sort of one-at-a-time fashion but they tarry here until all of the cells in the greater part of the tubule have reached this stage; then the nuclei of a given portion all proceed to the open spireme stage, shown in figures 11 and 12. These figures show only the chromatin which lies on the side from which the nucleus was observed. The chromatic material is again arranged in the peripheral portion of the nucleus and is segregated into the chromosomes which become somewhat massed in the center of the nucleus. The spindle next makes its appearance (fig. 13) and the chromosomes are drawn into the equatorial plate (fig. 14).

The mitotic figure represented in figure 15 shows the possibility of a tri-polar division. Such a condition may have been brought about by the formation of one of the spindles of the second division before that of the first division was completed. There is a small portion of the chromatin of this nucleus which is not involved in the mitotic figure. This portion is shown at *e.*, in figure 15 a, which is a drawing of what was seen at a lower level than that shown in figure 15.

The chromosomes in these nuclei are so small and so closely crowded together it is very difficult to determine their structure or their number. In one preparation, however, I obtained a ring-shaped appearance of the chromosomes (fig. 16). These forms were seen in the equatorial plate and also before the chromosomes had been arranged in the plate. In most of the preparations the chromosomes appear as mere granules. It may be that the ring-shaped forms were produced by the fixing reagent, which may have caused a swelling of the chromosomes. This result was not always obtained, however, by the same reagent.

When destaining is carried so far as to remove all the stain from the cytoplasm and the achromatic figure, the equatorial

plate may be shown to have a structure like that represented in figure 16 a. This was drawn from a section cut from the edge of the plate. Here it appears that the chromosomes are stretched as they are pulled apart. Strands of chromatin pulled out between the separating groups of chromosomes may be seen in the later stages of the anaphase. By more extensive destaining we may obtain what appear to be only the cores of the chromosomes as shown in figure 16 b.

In figures 17 to 22 various stages in the anaphase are represented. The interzonal fibers and the mid-body are very distinct in figures 19 to 21.

The second miotic division follows very soon after the first. The chromosomes become somewhat separated and are then drawn together again into the equatorial plate ready for the second division (figs. 23 to 25). Figure 26 shows the beginning and figure 27 the end of the anaphase. Here again the interzonal fibers and the mid-body are distinctly seen and a portion of the cytoplasm is definitely associated with each daughter nucleus. The nucleus of the spermatid is now organized and persists in a sort of resting condition for a comparatively long time. The centrosome may also be distinguished for a considerable time, but later I was unable to recognize it (figs. 28 to 32). A clear space surrounding the nucleus is also seen in these figures. The spermatid as it appears in figure 32 rests for a considerable period before any change towards the formation of the spermatozoon is observed. The boundaries between the cytoplasm of the different cells disappear and the nuclei come to lie in a sort of plasmodium.

The transformation of the spermatid into the spermatozoon

In serial sections of a single tubule we may trace every stage in the transformation of the spermatid into the spermatozoon, and since the two ends of the series are in opposite ends of the tubule and the intermediate stages lie in serial order between these ends, we may use the position of a seminal element in the tubule as a criterion for determining its relative stage in the

course of development. The first evident step in the transformation of the spermatid is the appearance of vacuoles in the cytoplasm next to the nucleus. These are small at first but by coalescing they soon form a large, clear vacuole on one side of the nucleus (figs. 33 to 38). Sometimes it appears that the vacuole may have arisen by the nucleus settling to one side of the clear space surrounding it as in figure 31. The nuclei, each with its accompanying vacuole, now lie in a common mass of cytoplasm. In the further development of these cells there are three parts which must be constantly borne in mind, namely, the nucleus, the vacuole (hereafter called the capsule) and the cytoplasm. We will take up certain stages in the differentiation of these three parts, and consider their relation to each other.

In figures 37 to 41 the shape of the nucleus may be somewhat modified by strains in the cytoplasm or by the crowding of the elements in the tubule. In these drawings there is no evidence of a granular or reticular structure, although such structure was made out in some preparations which were destained to a greater degree. In figure 37 it may be observed that the outer layer of the nucleus stains more densely than the inner portion. The nucleus in figure 38 contains a vacuole which does not take the stain. The cytoplasm surrounding the nucleus and capsule in figures 37 to 39 is nearly uniform in appearance, with probably a tendency to be a little more deeply stained near the nucleus. In figures 40 and 41 there is a concentration of a portion of the cytoplasm on one side of the capsule and bordering the nucleus. This is finely alveolar and stains more deeply than the rest of the cytoplasm. It may be that this patch of cytoplasm is seen in an earlier stage in figure 36 *c*, *a*. This portion of the cytoplasm crowds in between the nucleus and the capsule (fig. 42). About this time the capsule begins to take a brownish color when stained with iron-hematoxylin.

The origin and development of this portion of cytoplasm which appears on the side of the capsule and nucleus and wedges in between them is a striking feature in the development of the spermatozoon. Its behavior is well brought out in figures 45 to 48. In figure 45 we see this substance slipped in like a wedge

between the nucleus and the capsule, with a clear space between it and the nucleus. If the spermatid shown in this figure were rotated to the right through 90° so as to bring the outer surface of the wedge of cytoplasm on the side toward the observer, we would have the appearance presented in figure 46. If we should turn this through 180° so as to throw the wedge on the opposite side from the observer, the spermatid would appear as in figure 47 where just the tips of the crescent shaped wedge are seen. The tips of this crescent progress around the capsule along the boundary line between the nucleus and the capsule. At the same time the thick side of the wedge is reduced and the material is distributed equally around this border-line to form a complete ring, which viewed from *any* lateral direction, has the appearance shown in figure 48. At first the substance of the wedge is finely alveolar in appearance but by the time the ring is completed it seems to be uniform throughout and is stained black with iron-hematoxylin. It seems to be identical with the mitochondrial substance described by Koltzoff ('06).

After the mitochondrial ring is completed, the nucleus becomes widely separated from it and the capsule (figs. 50 to 52). This however is not always the case. In two preparations from which figures 33 to 35 and 37 to 43 were drawn, the nucleus remained fitted closely down on the capsule as shown in figure 43. As the two different conditions were obtained with the same fixing fluid it is hardly probable that the difference was caused by the fixing. The nucleus at this time loses the last trace of any granular or reticular structure and becomes uniform in its staining reactions, and somewhat reduced in size.

About the time the mitochondrial mass begins to slip in between the nucleus and the capsule, one or two deeply staining granules appear on the border line between the nucleus and the capsule (figs. 44 to 48). Koltzoff ('06) in his researches on the spermatogenesis in Galathea squamifera, has identified these granules with the centrosome. In my preparations of Menippe mercenaria I am able to distinguish the centrosome for some time after the second maturation division (figs. 28 to 32) but, in the later resting period of the spermatid and in the stages during

the origin of the capsule, I am unable to distinguish any granule which can with any certainty be identified with the centrosome. I shall call the structure developed from this granule, the 'central body.' I am unable to follow the development of two distinct granules although two could sometimes be clearly distinguished as shown in figure 44. Probably only the outer one is concerned in the development which is here presented.

This outer granule elongates (fig. 47) and becomes tubular (figs. 50 to 56). There soon appears at the outer end a vesicle which increases in size as the central body elongates. While the vesicle is still small there appears in its outer wall a flattened granule which is usually seen to be connected with the end of the central body by means of a fine strand as though it might have been derived from this body. As the central body increases in length and the vesicle enlarges, its outer wall approximates the outer wall of the capsule. The deeply staining substance in the outer wall of the vesicle now becomes connected with the wall of the capsule (figs. 56 to 58). A second vesicle now forms (fig. 59). These two vesicles become transformed into a tubule containing the central body. This tubule will hereafter be spoken of as the 'inner tubule.' At its outer end a ring of darkly staining substance is found (fig. 60). This seems to have been derived from the central body. At least a study of figures 54 to 60 may well suggest such an interpretation. The central body finally becomes reduced in diameter and appears to be a solid rod. It is not stained by thionin nor by safranin, but is readily stained with iron-hematoxylin. The inner tubule is stained green with safranin counter-stained with Lichtgrün; blue with thionin counter-stained with eosin; and black with iron-hematoxylin.

During this whole period the content of the capsule shows an increasing affinity for chromatin stains. It is colored brown with iron-hematoxylin. In some series a sort of ring-shaped cloud appears in the capsular contents. At first it is near the outer wall but gradually it contracts towards the vesicle at the end of the central body and finally settles in the wall of the tubule when that structure takes its final form. With Delafield's hematoxylin the contents of the capsule is readily stained, and with

safranin it takes a dull red color. In the early stages of development the content of the capsule is stained green when the preparation is treated with the safranin and Lichtgrün combination, but in the later stages the green is masked by the red. In stages represented in figures 53 to 55 a sort of foam or alveolar structure can sometimes be observed in this substance.

While the capsule and the structures within it are assuming their mature form, the nucleus has become less densely stained and settles down upon the capsule like a cap (figs. 52 to 59). It becomes thin in the center so that its final shape is that of a cup with a rounded, thin bottom and a thickened rim. This thickened border fits upon the mitochondrial ring so that in the mature spermatozoon it is not possible to distinguish it from that ring.

Protoplasmic rays or pseudopodia develop from the rim of the cup. I have been unable to determine whether they arise from the mitochondrial substance or from the nucleus.

The spermatozoon

We may now consider the structure of the mature spermatozoon. Figure 61 is a drawing of a spermatozoon taken from the seminal receptacle of the female and killed in the vapor of osmic acid, then stained with gold chloride after treatment with formic acid. We observe the nuclear cup (*n.c.*) from which the pseudopodia (*ps.*) arise. Inside the cup is the spherical capsule (*c.*) within which there is the capsular cavity (*c.c.*); and the inner tubule (*i.t.*) with its cavity divided into the inner tubular cavity (*i.t.c.*); and the outer tubular cavity (*o.c.*). Running through the inner tubular cavity and through the wall of the inner end of the tubule to the bottom of the capsule we see the central body (*c.b.*). Figure 62 was drawn from a live spermatozoon in 4 per cent KNO_3, and figures 63 to 65 are from spermatozoa mounted in the serum of the crab's blood. Movements of the blood have bent the pseudopodia of these spermatozoa. Otherwise they have more nearly the natural shape and propor-

tions than those shown in figures 61 and 62. The diameter of the capsule of these spermatozoa is about 3.8 μ and the pseudopodia are sometimes as much as 7 μ long.

Spermatozoa in the deferent duct

The mature spermatozoa pass from the tubules of the testis into the deferent duct. The latter is a long, extensively folded, tube lined with glandular epithelium. The spermatozoa form a common mass when they enter this tube, but the secretion formed by its lining flows in among them and separates them into groups. The secretion surrounding each group then hardens and so forms a membrane, so that finally there are an immense number of capsules containing the spermatozoa. These capsules are known as spermatophores. In this condition the spermatozoa are transferred to the seminal receptacle of the female crab.

Summary and discussion

In this study of spermatogenesis in Menippe mercenaria the principal points brought out are as follows:

1. There is a single row of cells which persists on one side of the testicular tubule and gives rise to successive batches of spermatozoa.
2. The spermatogonia divide without the formation of a spireme. The chromatin simply aggregates into chromosomes which are then gathered into an equatorial plate.
3. The maturation divisions follow one another quickly. They are preceded by spireme formation and a long period of synapsis.
4. There also seems to be a relatively long resting stage after the nucleus of the spermatid is formed before the transformation into the spermatozoon begins.
5. In the transformation of the spermatid, three structures must be considered, namely, the nucleus, the capsule and the mitochondrial ring.
6. The nucleus becomes uniform in consistency, reduced in size and cup-shaped.

7. The capsule arises in the cytoplasm as a clear vacuole which may be stained with Lichtgrün. Its content is gradually changed to have a greater affinity for chromatic stains.

8. From a granule on the proximal side of the capsule the central body develops into the capsule. At the distal end of this body a vesicle arises, which is changed into the inner tubule.

9. The mitochondrial substance is segregated from the cytoplasm and deposited as a ring between the nucleus and the capsule.

Some of the theoretical questions connected with the development and structure of the spermatozoa of the decapods will be taken up at the end of this article. At this point I wish to say that the above description is in agreement with the principal observations made by Grobben ('78), Gilson ('86), Sabatier ('93), Brandes ('97) and Koltzoff ('06). These authors have all seen the same general structures and transformations. They all describe a nucleus which, during development, is modified in its staining reactions, reduced in size and often flattened or otherwise changed in its shape. They do not disagree as to which part of the cell is the nucleus. They likewise describe a vesicle which arises in the cytoplasm either against the nucleus or close to it, and they mention the substance of cytoplasmic origin which appears between the nucleus and the vesicle. Most of them see a structure like the central body and describe the inner tubule. There are many variations in the detail of the development of these last two structures, and different species seem to differ widely in this respect. There is much disagreement concerning the destiny of the nucleus and the origin and nature of the substance in the capsule. These points of disagreement do not however affect the statements I have made concerning the structure of the mature sperm. It is with this structure that we have to do in the further course of the present investigation.

3. COPULATION

We now come to the question of the transfer of the spermatophores from the body of the male to that of the female; from the deferent duct to the seminal receptacle. We therefore turn our attention from the sperm itself to some of the habits of these crabs. Menippe mercenaria lives in crevices under or between the rocks, or in burrows which it digs in the mud along the shore a little below low water line. Usually one crab is found in each burrow, but occasionally, and even frequently in the month of August, a male crab will be found guarding a hole in which there is a female. Sometimes the female thus found has a soft shell. If its shell be hard it molts within a few days after being brought into captivity. On August 17, a female with a soft shell and male crab which had been taken from the same hole about noon, were placed together in a compartment of a floating cage. At 5:45 P.M. they were observed to be copulating. On being disturbed they separated. Their behavior was then observed while copulation was resumed. The most significant point with regard to this behavior was the apparent care with which the male acted in order to inflict no injury upon the soft, delicate shell of the female.

During copulation the spermatophores are transferred from the deferent duct to the portion of the seminal receptacle which is lined with chitin, where they are deposited in a very compact mass. Here they remain until the next spawning of eggs. Only a portion of the spermatozoa are used for the fertilization of any one batch of eggs. One crab, kept by itself in a compartment of a floating cage for sixty-nine days during the summer of 1911, spawned six times and apparently all of the eggs in the six different batches of 500,000 to 1,000,000 eggs each, were fertilized and developed normally.

4. SPAWNING HABITS

The spawning habits and the development of this crab will be discussed in a later paper. Here we will present only such points as are necessary in order to make it clear how the stages in the entrance of the sperm and fertilization are obtained.

When a female is ready to lay a batch of eggs she assumes an upright position and holds the abdomen out from her body so that it and the exopods of the abdominal appendages form a basket into which the eggs are run. They there become attached to the hairs of the endopods of the appendages and pass through the embryonic stages of their development, which requires from nine to thirteen days. The eggs then hatch and the larvae escape. The female then cleans off the egg-shells and their stalks from the hairs of the pleopods and, after one day to three weeks, she spawns again. Eight days is a very common length for the period between the hatching of one batch of eggs and the spawning of the next. With these facts in mind I made a large floating cage with fifty compartments and collected a large number of females with eggs and placed one in each compartment. After the eggs of several of these had hatched so that there were some fifteen crabs without eggs I kept these under almost constant observation, day and night. When one assumed the position ready for spawning it was naturally supposed to contain eggs which were mature if they were not already fertilized. Before describing the process of fertilization we should consider briefly the structure of the genital organs of the female.

5. THE REPRODUCTIVE ORGANS OF THE FEMALE

Figure 121 is a diagrammatic representation of the ovary and one seminal receptacle and oviduct of this crab. The ovary is an *H*-shaped tube, the lumen of which opens directly into the seminal receptacle at a point a little posterior to the cross connection of the *H*. The eggs are produced in the wall of this tube and when mature are set free in the lumen.

The seminal receptacle is composed of two parts, a glandular portion (figs. 121 and 122, *g.*) into which the ovary opens and a portion lined with chitin (figs. 121 and 122, *c.*) from which the oviduct leads to the third segment of the sternum. The spermatophores are stored in the latter division. The cavities of the two portions communicate through a large opening (fig. 121, *o.*) in the chitinous lining. Just before the crab molts, the glandular portion secretes a mass of gelatinous material which

greatly distends it (fig. 122) and the spermatophores are by some means transferred to this part of the receptacle where they lie in the mass of jelly. This prevents them from being lost at the time of molting when the chitinous lining is shed. Whether they are returned to this part of the receptacle after the molting has not been determined. The glandular part of the receptacle is rapidly reduced after the shell is shed, but I do not know what becomes of the secretion. During spawning the glandular portion is very much contracted (fig. 121) so that it is little more than a tube connecting the ovary with the chitinous receptacle. There is one possibility which may be mentioned here; the glandular receptacle may secrete a semi-fluid substance and then, by contracting, force the spermatozoa into the lumen of the ovary just before spawning begins. As I shall show later, the spermatozoa are transferred to the ovary. This however is only a conjecture as to the method of the transfer. The only time at which the receptacle is known to be actively secreting a substance is just before molting and it may simply be a device for retaining the spermatophores at the molting period.

If a crab that has just begun to lay its eggs be opened, the lumen of the ovary and the oviduct will be found to be full of eggs. Some eggs were taken from the lumen of the ovary with a sterilized pipette and placed in filtered sea-water. Since these developed into embryos it is evident that fertilization takes place in the ovary. Sections were made of eggs taken from the lumen of the ovary and from the oviduct and from these the phenomena of fertilization were observed, but we will return to this later.

6. THE BEHAVIOR OF THE SPERMATOZOA

The spermatozoa of this crab are so very minute, the eggs so relatively large and opaque, and the conditions for sperm entrance so difficult to reproduce on the microscopic slide, I did not see the living spermatozoon enter the egg. It is easy, however, to interpret the structures seen in sections of eggs taken at spawning time, after one has observed the behavior of the spermatozoa under certain experimental conditions. We will proceed, therefore, to a description of this behavior.

Methods of study

Koltzoff ('06) by his careful analysis of the effects on the sperm of solutions differing in osmotic pressure, has cleared up many of the mysteries of the decapod sperm. According to his researches, the spermatozoa maintain their normal form in solutions of salts having the same osmotic pressure as sea-water. He also found that 5 per cent KNO_3, 2.8 per cent NaCl, 4.25 per cent $NaNO_3$, 18.5 per cent $MgSO_4$, 7 per cent glycerine and 25.65 per cent sugar solutions are isotonic with sea-water. Solutions of these salts at a lower concentration cause a deformation of the spermatozoa.

For my studies, solutions of KNO_3, NaCl and $NaNO_3$ were used. The spermatozoa taken from the seminal receptacle and placed in solutions of these salts isotonic with sea-water would remain many days without perceptible change. When they were placed in weaker solutions of these salts transformations occurred. In studying these changes I proceeded as follows: Spermatozoa from the seminal receptacle were placed in the serum of the crab's blood or in the isotonic solutions of KNO_3, NaCl, or $NaNO_3$. In these solutions they were transferred to the slide, covered and examined under the high power of the microscope. Then, by placing a weaker solution of one of the salts at the edge of the cover-glass and allowing it to diffuse underneath, a slow change in the form of the spermatozoon was obtained. This change was thus followed in detail. It is to these changes that we will now turn our attention.

By referring to figure 61, we may again call to our minds the normal condition of the mature spermatozoon which consists of a chitinous capsule, set in a protoplasmic cup. The capsule contains a tubule with an inner and outer cavity and, running through the inner cavity of the tubule, is the central body, the proximal end of which rests on the wall of the capsule.

Changes in the nuclear cup

When solutions with a lower osmotic concentration than sea-water come in contact with the nuclear or protoplasmic cup it becomes thicker and the pseudopodia are withdrawn so that the outline of the spermatozoon, viewed from the top or bottom of the cup, is circular instead of star-shaped. The disappearance of the pseudopodia proceeds by a swelling at the base while the outer portion tapers very gradually to an extremely fine point (compare fig. 62 with 63 and 65). As the base widens out still farther the rays are reduced to a very fine thread, which either breaks off or is contracted into the body. When the pseudopodia break loose from their attachment the whole spermatozoon is apt to move suddenly and then be borne away if there be any currents in the containing fluid. This sudden movement probably results from some of the pseudopodia breaking loose slightly before the others. This rather than the explosion of the capsule may be the explanation of the 'springing of the sperm' discussed by Koltzoff ('06). This rounding up of the protoplasmic portion of the sperm is apt to be completed before any change takes place in the capsule. Sometimes, however, the capsule may be completely changed before the disappearance of the pseudopodia. Probably, in rapid explosion, the two take place simultaneously.

Changes in the capsule

For the interpretation of the entrance of the spermatozoon into the egg the transformation of the capsule is much more important than the changes in the protoplasmic cup. We will therefore follow the capsular changes very carefully. The first change is the out-pushing of the outer cavity of the inner tubule (compare fig. 61 with 67). Here it is evident that the wall of the outer cavity of the inner tubule has been everted, while the wall of the inner cavity (fig. 61, *i.t.c.*) has been stretched. It is difficult to see just what change has taken place at this time in the central body. In some instances it appeared that it had been lengthened, and in some specimens I thought the end of

it could be seen at the summit of the out-pushed portion. It may be that the lengthening of this body is the force that turns this distal cavity inside out.

In the next step of the capsular inversion the thick covering of the out-pushed part shown in figure 67 becomes turned out laterally so as to form a collar (fig. 68, *r.*) and the inner tubule becomes farther everted. The collar formed at this stage persists unchanged throughout all the further modifications of the capsule. The central body may now become greatly increased in length so that it projects beyond the out-turned part of the inner tubule (fig. 69, *c.b.*, also figs. 70 to 72). From this stage on to the completion of the eversion there is little further increase in the length of this axis. The everted portion of the inner tubule, however, swells out more and more (figs. 77 to 79). The transition from the condition shown in figures 71 and 76 to that in figures 77 to 79 is brought about by a further eversion of the inner tubule. The part of the inner tubule involved in this second definite eversion is probably marked by the funnel-shaped portion in figure 76. The portion of the everted wall, derived from the part of the tubule turned by this second eversion, is indicated by the granule *g* in figure 77. At this stage there is another pause while the out-turned part continues to swell.

Finally, the tension becomes so great that another portion of the inner tubule is everted and, as it turns, the wall of the capsule is also turned through the collar formed in the early stage of the process of eversion. This last eversion is shown halfway completed in figure 80, and the completed process in figure 81. In the latter figure the central body stands on the apex of the eversion and the inverted capsule (*inv.c.*) is above the collar (*r.*). In dilute solutions of the salts used, the protoplasmic portion, which contains the nucleus and mitochondrial substance, swells up to a spherical body as shown in figure 82. Often one finds on the slides, bodies like the one represented in figure 83. It is evident that these are exploded spermatozoa from which the everted inner tubule has disappeared, leaving the central body (*c.b.*), the inverted capsule (*inv.c.*), the collar (*r.*), and the shrunken nuclear cup (*n.c.*).

Changes in the central body

We now return to a more complete consideration of the behavior of the central body and the part that it plays in the explosion of the spermatozoon. These spermatozoa are so very small it is difficult, in many cases, to distinguish the central body, especially in the live, unstained material. Some significant facts, however, have been observed. As is shown in figure 61, the central body is composed of two distinct parts, the distal part within the cavity of the inner tubule and a proximal part connecting the inner end of the tubule with the wall of the capsule. Whether the central body projects into the outer cavity of the inner tubule or ends against the shelf separating the inner and outer cavities of the tubule, was not definitely determined, but the latter seems to be the case.

In figure 66, which was drawn from a spermatozoon in the tubule of the testis, fixed in Worcester's fluid and stained in iron hematoxylin, the central body projects through the apex of the capsule. This condition may have been brought about by an elongation of the central body or by a shrinking of the capsule. In either case it indicates that the central body is more or less rigid. One should notice also that the fixing fluid has caused a shrinking of the nuclear cup, so that it is now more like a saucer than a cup.

In figures 69 to 72, which were drawn from living spermatozoa, the central body projects beyond the everted tubule like a rigid rod, giving the impression that its elongation may have had something to do with the stretching of the tubule and the lengthening of that axis of the spermatozoon. The idea that the central body is somewhat rigid is further supported by its appearance in figures 73 and 74, where it stands out above the everted tubule. The same condition is produced in figures 81 and 83. Probably the strongest evidence in favor of the rigidity of this structure is found in figure 75, where, in lengthening, it has pushed backwards through the wall of the capsule and pushed the nuclear cup away from the wall of the capsule.

There are some indications that the central body is not firm but a plastic, semifluid substance. This is supported by the fact that it sometimes glides out through the inner tubule at stages such as that shown in figure 76 and adheres to the surface of the everted tubule in one or more amorphous masses (figs. 77, *g.*, 79 and 82). This condition may have been brought about by a degeneration of the body as a result of keeping the spermatozoa in the serum of the blood or in salt solutions. Sometimes in unexploded spermatozoa the central body adheres to one side of the tubule instead of standing in the center, and it may be that it was only in such cases as this that it adhered to the everted wall of the tubule.

Dynamics of eversion

We may now consider the forces involved in the turning of the tubule and capsule inside out. We may divide this inquiry into two questions: (1) What are the *external* conditions necessary to initiate and carry on the process? (2) What are the *internal* conditions which respond to the external ones and determine the nature of the process?

As stated above, a decrease in the osmotic pressure of the medium in which the spermatozoa lie, will cause the eversion. Unexploded spermatozoa, taken from blood serum and placed in 5 per cent KNO_3, do not explode; placed in 3 to 4 per cent KNO_3 they take the forms shown in figures 67 to 70; in 2 to 3 per cent KNO_3, the forms in figures 70 to 72 and 76 are obtained; in 1.5 to 1 per cent KNO_3 the eversion proceeds to the stages shown in figures 77 to 82. Like results were obtained by treating the spermatozoa with dilutions of 2.8 per cent NaCl or 4.25 per cent $NaNO_3$. Not all the individuals are equally affected by these solutions. Many of the spermatozoa retain the unexploded conditions of the capsule for a long time in a 2 per cent KNO_3 solution, and often none of them attain to the stage represented in figure 82 when treated with 1 per cent KNO_3.

Spermatozoa kept for several days in 2.8 per cent NaCl exploded when transferred to 4.25 per cent $NaNO_3$. Here we had

an explosion when they were transferred from a solution of one salt to that of another with equal osmotic pressure. Fresh spermatozoa do not explode when placed in 4.25 per cent $NaNO_3$; therefore the spermatozoa must have been changed by the NaCl, or the presence of these two salts must have had an effect that neither had when acting alone. To determine the factors here acting will require further experimentation.

Some of the spermatozoa explode whenever they are transferred to a slide and covered with a cover-glass. The cause of such explosions was not determined. Koltzoff found that mechanical pressure would cause the explosion of the spermatozoa of some Decapods. I failed to produce any explosion by pressing on the cover-glass of a preparation containing them. Koltzoff ('06) made extensive researches to find some specific stimulus that would cause a certain definite explosion which he believed to be the normal one but failed to find one. It appears, however, that a careful investigation of the conditions which initiate the process, followed up by an analysis of the conditions which may increase the pressure within the capsular cavity (fig. 61, *c.c.*), would throw valuable light on this subject. My researches have been concerned with the exact changes which occur in the spermatozoon, rather than with the conditions that cause the changes.

The second question, the one concerning the internal conditions which determine the response of the spermatozoon to the external conditions, may now be considered. What is there in the spermatozoon which may react to a decrease of the osmotic pressure of the solution which surrounds it? An examination of figures 68 to 82 clearly shows that it is the capsular cavity which increases in size. It must therefore contain a substance which is isotonic with sea-water and with the blood of the crab and which absorbs water when placed in any solution which is of a lower concentration. This water is doubtless taken in through the wall of the inner tubule, which seems to be semipermeable, while the outer wall of the capsule is probably impervious.

Another striking feature of the explosion is the remarkable extensibility of the wall of the inner tubule which is everted

to form the wall of a structure many times larger than the capsule. The central body must also be considered as one of the structures taking a part in the explosion of the capsule. We have therefore three changing structures, a swelling mass, a stretching membrane, and an elongating body, each of which take a part in determining the form of the inversion. To these must be added two structures which do not change and are resistant in their nature. These are the wall of the capsule and the collar surrounding the hole in the capsule, through which the tubule is everted. Let us now follow the interaction of the forces involved in the behavior of these changing structures. For this purpose we shall divide the explosion into four stages.

Stage 1. The eversion of the outer cavity of the tubule (*fig. 67*). Two forces probably take part in this, the pressure in the capsular cavity and the elongation of the central body.

Stage 2. The elongation of the everted outer cavity (*figs. 68 to 71 and 76*). This results in the formation of the collar (fig. 68, *r.*). Here again two forces may be involved, the swelling of the material in the capsular cavity and the further elongation of the central body which stretches the portion of the inner tubule which bounds the inner tubular cavity. The fact that the everted portion is sometimes longer in the axis through which the central body passes, indicates that this body may be exerting an out-pushing force. If this be the case, we have here an elastic body which has become active by being released from compression; that is, the central body elongates like a coiled spring. This action is fully discussed by Koltzoff. The pressure in the capsular cavity is sometimes shown by the squeezing of the central body out through the outer end of the tubule when it has lost its resistant properties.

Stage 3. The second eversion of the inner tubule (*figs. 77 to 79*). From the condition shown in figures 71 and 76, the increasing pressure in the capsular cavity causes the wall of the everted tubule to swell to the form shown in figure 72. Finally, the pressure becomes so great that the ring which formed the division between the inner and outer tubular cavities (fig. 61, *i.t.c.* and *o.c.*) gives away and a part of the tubule bounding the inner

tubular cavity becomes everted. Portions of the central body often adhere to the wall of the tubule and are carried outward and so mark the extent of this second eversion (fig. 77, *g.*).

Stage 4. The third eversion of the tubule, accompanied by the inversion of the capsule (*figs. 80 to 82*). The internal pressure continues to increase, as is shown by the bulging out of the walls of the everted portion (figs. 78 and 79). This brings a strain upon the axis in which the tubule and central body lie. This tends to stretch these structures as is shown by the incurving of the apical wall of the everted portion in figures 78 and 79. This causes the base of the everted part to press on the sides of the capsule. This pressure on the sides of the capsule, together with an up-pulling along the line of the central body, results in turning the capsule through the collar when the last section of the tubule is everted. In figure 82 the portions of the everted wall contributed by the second, third and fourth stages of the eversion are probably indicated by the granules g^1 and g^2. Thus we see that the whole transformation may be explained by the increase of pressure in the capsular cavity together with tensions along the line of the inner tubule and the central body.

Efforts were made to reverse this process by placing spermatozoa in very concentrated solutions of the salts used. The only effect of this treatment was a shrinking of the everted portion, which would again swell up and the process of eversion continue when dilute solutions were again used. After the explosion had reached the stage presented in figure 82 the only part affected by concentrated solutions was the protoplasmic portion at the bottom. It is also true that this is the only part that takes methylene blue, methyl green or thionin stains when these are applied to the living spermatozoa. It was rather surprising that the contents of the everted capsule were not stained by these stains. Sometimes a few granules can be seen in this cavity.

Figures 73, 74, 84 and 85 were made from spermatozoa which had been kept in a 5 per cent KNO_3 solution for fifty-one days. We observe here that partial explosion had taken place. Those shown in figures 84 and 85 had reached the stage corresponding to figure 70. When these were treated with a solution of lower

concentration the eversion continued, but the wall of the part already everted seemed to be hardened, so that it made an elongated collar through which the further inversion took place.

The effect of reagents on the everted spermatozoa

After trying several fixing reagents it was found that for imbedding and cutting the crab's eggs Morgan's fluid gave decidedly the best results. This fluid is a saturated solution of picric acid in 30 per cent alcohol, to 100 cc. of which 2 cc. of H_2SO_4 are added. Spermatozoa in various stages of the process of inversion and those in the normal mature condition were mounted under the microscope and killed with this fluid in order to determine its effect upon them. A considerable amount of shrinking was observed, but no decided change in the relationship of the parts seemed to take place. Figures 86 and 87 were drawn from spermatozoa exploded in distilled water, fixed in Morgan's fluid, stained in thionin and eosin, dehydrated in alcohols, cleared in xylol and mounted in balsam. The general relations of the parts is the same as in figures 81 and 82. The space between the everted tubule and the inverted capsule seems to have shrunk relatively more than the inverted capsule. Having now observed the behavior of spermatozoa under experimental conditions we may proceed to our observations concerning the extrance of the spermatozoon and the process of fertilization.

7. THE ENTRANCE OF THE SPERMATOZOON

Eggs were taken from the lumen of the ovary just after the crab began to spawn, and were fixed in Morgan's fluid, imbedded in paraffin and sectioned. A microscopic examination of these eggs showed the spermatozoon in the act of entering the egg. The best stain for the study of these sections is thionin, for it stains the chromosomes in the mitotic figure of the nucleus of the egg and the nuclear cup of the spermatozoon a deep blue. It stains the everted portion of the spermatozoon faintly and the food material, cytoplasm and egg-shell are unstained or only

faintly stained. This treatment makes it possible to find these minute structures in the relatively immense egg. After one has become familiar with these structures and their position on the egg it is possible to find them quite readily with other stains such as Delafield's hematoxylin, iron-hematoxylin, or safranin and Lichtgrün.

The relation of the spermatozoa to eggs taken from the lumen of the ovary is shown in figures 88 to 92. Spermatozoa in the same condition are also found in eggs taken from the oviduct as is shown in figures 93 and 94. Now, when these figures are compared with figures 86 and 87, which have been treated with the same fixing reagents, it is evident that it is the everted portion of the spermatozoon which has gone through the shell of the egg. The nuclear cup (*n.c.*, figs. 91 and 92) is on the outside of the shell. The everted tubule forms a vesicle within which one sees the inverted capsule (*inv.c.*, fig. 91). Hereafter I shall call this everted tubule and capsule, the 'sperm-vesicle.' At the inner-end of this sperm-vesicle the ejected central body may be seen (figs. 88, 91 to 94, *c.b.*). That the part which remains outside is the nuclear cup with its radiating pseudopodia can be more clearly seen by a surface view of the structure as it lies upon the egg, such as is presented in figure 95. Furthermore, the staining reactions are in accord with those observed in the mature spermatozoon and the artificially exploded ones and are as follows:

Thionin and eosin	the part outside of the shell, blue the part inside of the shell, red
Safranin and Lichtgrün	part outside of shell, red part inside, green mixed with red
Iron-hematoxylin	part outside of shell, black part inside, brown except the central body which is black

Koltzoff ('06) claimed that in certain Decapods the spermatozoa settled on the egg with the nuclear cup towards the egg and the capsule pointed away from the egg. He was also of the opinion that the rebound from the explosion of the capsule was sufficient to drive the nucleus into the egg. On eggs taken

from the ovary of Menippe mercenaria, I found a few spermatozoa attached, as shown in figure 96, with the nuclear cup next to the shell of the egg. That the eversion of the capsule does not force the nucleus through the shell in this case is shown in figure 97, where a spermatozoon has exploded with the nuclear cup against the egg. So far as my observations go there is no evidence whatever that the eversion of the capsule causes any sudden movement of the spermatozoon body as a whole.

The number of spermatozoa which have pierced the shells of the eggs is much greater for eggs taken from the oviduct than for those taken from the lumen of the ovary. The number was counted in a few eggs which had just been spawned and the number per egg was as follows: 28, 44, 52, 52, 54, 71, 73 and, in one exceptional case, 679.

So far there seems to be no doubt as to the behavior of the spermatozoon in entering the egg, but we may ask: Is this the final stage in the entrance of the spermatozoon? Is not the nuclear cup drawn through the shell at a later stage? If not, what becomes of it?

That the nuclear cup does not enter the egg, but falls off is shown in figures 98 to 101. Here we see that the nuclear cup has moved away from the egg-shell and that a strand of some substance, by which it was probably attached to the bottom of the capsule, is drawn out with it. Sometimes the nuclear cup breaks loose from the strand and leaves it projecting through the shell into the capsule (fig. 100). In eggs taken from the oviduct or from the pleopods just after spawning, large numbers of sperm-vesicles are found sticking to the inside of the shells after the nuclear cup has fallen off (fig. 101). It is very clear then that, in most cases at least, the nuclear cup does not enter the egg. But does it thus fall off from the particular spermatozoon which fertilizes the egg, or only from those which have failed to perform the work of fertilization? This question can best be answered by a further study of the events of fertilization.

8. FERTILIZATION

In eggs taken from the oviduct or just after leaving it, many sperm-vesicles may be found lying on the edge of the cytoplasm as shown in figures 102 and 103, while *one* is found down in the cytoplasm (figs. 104 to 108). Here it is evident that the movement of the cytoplasm has carried the vesicle below the surface. Before the sperm-vesicle enters, there is a layer of cytoplasm just inside of the egg-shell. The rest of the egg is filled with spherules of food material, in the interspaces of which the cytoplasm extends from the peripheral layer, by fine strands, all through the egg. The fact that the spherules of food move apart and a small mass of cytoplasm accompanies the sperm-vesicle into the egg, is best explained by supposing that the first vesicle which comes in contact with the cytoplasm, initiates a flowing movement of the latter along the inner surface of the shell, from all sides towards the newly entered vesicle. The cytoplasm, moving thus along the inner surface of the shell towards one point, would be deflected in towards the center of the egg, and would tend to carry the vesicle in with it. We may suppose further that, when the cytoplasm has once responded to such a stimulus, its physiological state is so changed that it will not respond to another. As a result only one vesicle becomes imbedded in the cytoplasm of the egg where it is to be transformed into the male pronucleus.

The vitelline membrane (fig. 104, *v.*) is formed just after the entrance of the sperm-vesicle into the cytoplasm and the vesicles which failed to enter, lie between it and the shell of the egg. The first polar body (fig. 114), which is cast off while the eggs are passing through the oviduct, is also found between the vitelline membrane and the shell.

The first step in the transformation of the sperm-vesicle into the male pronucleus is a thickening of its lateral walls. This may be observed in figures 105 to 111. Accompanying this there is an increase of affinity for the stains used (thionin and Delafield's hematoxylin). Next, there seems to be an extrusion of the old capsular wall which, if we recall the method of the ever-

sion of the spermatozoon, we know forms the inner lining of the sperm-vesicle. The discarding of this capsular wall was not clearly made out, but figures 109 and 110 indicate that such a change takes place. In eggs taken soon after spawning the sperm-vesicle has increased considerably in size and the wall has taken on a vesicular appearance. This is shown in figures 110 and 111, which show the sperm-vesicle in two different aspects. The cavity of the vesicle gradually disappears, leaving only a notch on one side (figs. 108, 109, 110 and 112). Figure 112 was about an hour old. In eggs two hours old the vesicle has taken on the appearance of an ordinary nucleus containing granules of chromatin (fig. 116). At this time it has gone about one-fourth of the distance from the circumference to the center of the egg (fig. 117).

We will now turn our attention to the egg-nucleus. When the eggs are set free in the lumen of the ovary, just before spawning begins, the spindle for the first maturation division is already formed and its long axis is parallel with the surface of the egg (fig. 113). As the egg passes through the oviduct the spindle turns to a position perpendicular to the surface of the egg and the first polar body is cut off. This is shown in figure 114, which is from an egg that has just passed out of the oviduct. Efforts were made to count the chromosomes just after this division. Twenty-five to twenty-eight were counted. Thus the double number would be fifty to fifty-six. They are however so small and placed so closely together that it is difficult to distinguish them accurately.

Between one and one-and-a-half hours after spawning the second maturation division takes place. The second polar body is apparently not cut off, but remains in the egg where it degenerates and is absorbed by the cytoplasm.

The female pronucleus is now formed and proceeds to the center of the egg, where it meets the male pronucleus. Figures 115 and 116 are drawn from nuclei fixed at two hours and fifteen minutes after spawning. At this time it is not possible to tell which is the male and which the female pronucleus unless the

slightly concave side of the one shown in figure 116 indicates that it is related to the nucleus shown in figure 112. In this case it would be the male pronucleus. The nuclei have grown rapidly and continue to do so until they reach the center of the egg. Their contents are finely granular. These granules increase in size as the nuclei become larger. Figures 118 and 119 show the position of the nuclei four hours after spawning and figure 120 (from an egg six hours old) shows them lying side by side in the center of the egg. They have become elongated and many times larger than they were when first formed. From the above description of fertilization it is evident that the nuclear cup takes no part in the formation of the male pronucleus, for the latter is derived from the sperm-vesicle which is the inverted capsule.

This completes the description of the structure and behavior of the male cells in the stone crab. We have here something unique in the method by which the sperm enters the egg and something exceptional in the phenomena of fertilization. These observations raise several theoretical questions, some of which we will now briefly consider.

9. DISCUSSION

We have here a case in which an infolded vacuole which arose in the cytoplasm is everted through the shell of the egg and fertilizes it. How may such an event be brought into harmony with the existing theories concerning the chromosomes? In all other cases of fertilization the nucleus with its chromosomes or at least its chromatin is considered the essential thing; the bearer of the paternal qualities to the egg. The part that they play in the theories concerning heredity is too important and useful to be lightly discarded. But, granting all that is claimed for the chromosomes, we are nevertheless face to face with the fact that in most cases they disappear during the telophase and are reformed in the next prophase of cell division. Between the miotic divisions they can be followed from one spindle to the next and in some other cases, some investigators have claimed to have

been able to observe the continuity of individual chromosomes from one division of the cell to the next one. But these are exceptions. The problem of the origin of the chromosome is a real problem. For many reasons, in our final analysis, we must go back of the chromosome. So without attacking the proposition that chromosomes are the means for distributing the hereditary elements at the time of division, we may take up the question of the origin of the chromosome before division.

The phenomena described in this paper force us to consider this question if we are to bring the fact concerning fertilization in this crab in line with existing theories. Does any of the chromatin from the nucleus of the spermatid enter the egg? We have shown that it is the substance in the wall or in the cavity of the capsule that enters and fertilizes the egg. Now is there any evidence that the chromatic substance in the nucleus is transferred to the capsule during spermatogenesis?

Grobben ('78) claimed that the capsule is derived from the nucleus of the spermatid. He described a change in the consistency and a reduction in size of the nucleus which occurred simultaneously with the development of the capsule. He seemed to be of the opinion that the nuclear material was transferred by diffusion from the nucleus to the capsule.

Herrmann ('90) suggests that when one follows the parallel transformations of the capsule and the nucleus, one gets the impression that there is a sort of migration of the chromatic substance from the nucleus to the capsule.

Brandes ('97) found two substances in the nucleus of the spermatid. One was stained blue with methylene blue, the other red with acid fuchsin. The latter settles to one side of the nucleus and then passes out into the cytoplasm. The later workers, Koltzoff ('06) and Spitschakoff ('09), describe no such process.

In my own investigations I have noted a decrease in affinity for chromatic stains and in the size of the nucleus. The capsule, on the other hand, showed an increasing affinity for iron-hematoxylin and safranin. These facts suggest a transfer of nuclear material.

Finally, if we may accept the views of Stauffacher ('10) and Derschau ('11), that basichromatin is derived from oxychromatin, the former being deposited from the latter, we may postulate a theory for the explanation of the phenomena of fertilization in this crab. I do not claim that the facts establish the theory; they only suggest it. Some of the basichromatin in the nucleus of the spermatid is dissolved by the oxychromatin and transferred to the capsule. After the capsule is everted into the egg and has entered the cytoplasm of the latter, the basichromatin is redeposited and thus the granular structure of the male pronucleus appears. It may be possible to explain the number of chromosomes which appear, by supposing that there are a certain number of different kinds of molecules which are deposited out of the oxychromatin and that these have such an affinity for each other that they are aggreagted into a definite number of groups, or they may be of such a structural nature than they can fall only into certain groups. Of course, I claim for this only that it is a possible explanation of phenomena which are apparently not in accord with the conception of an individual continuity of the chromosomes.

10. SUMMARY

1. The seminal elements in Menippe mercenaria arise from a single row of primary spermatagonial cells which persist along one side of the testicular tubule.

2. The tubule is divided into three or four regions by longitudinal partitions composed of epithelial cells. The seminal elements in the division next to the row of primary spermatogonial cells are younger than those in any other division. The region on the opposite side contains mature spermatozoa. The seminal elements in one end of a given division are further along in their development than those in the other end.

3. The spermatogonial nuclei lie in a common cytoplasmic mass and multiply irregularly without the formation of a spireme.

A spireme and synapsis occur in connection with the first miotic division. The second mitotic division follows soon after the first.

4. In the mature spermatozoon the protoplasmic portion, containing the nucleus, is cup-shaped. From the rim of the cup pseudopodia project like the rays of a star. There is a capsule half-imbedded in the cup. An inturned tubule is connected with an opening in the distal portion of the capsular wall, and a rod-like central body arises from the proximal side of the capsule and projects into the inner tubule.

5. In the transformation of the spermatid, the nucleus becomes uniform in consistency, reduced in size and cup-shaped. A mitochondrial ring is formed between the nucleus and the capsule. The capsule arises as a vacuole in the cytoplasm. In the course of its development it shows an increasing affinity for nuclear stains.

6. The central body develops from a granule which appears on the proximal side of the capsule. The inner tubule is formed from two vesicles which arise at the distal end of the central body.

7. Hypotonic solutions of various salts and possibly other stimuli cause a lengthening of the central body, an eversion of the inner tubule and an inversion of the wall of the capsule.

8. When the spermatozoa come in contact with the egg under normal conditions, the capsule is usually applied to the shell of the egg and the nuclear cup is directed away from the egg. In this position eversion takes place and the ejected central body, the inner tubule, and the capsule with its contents are thus turned through the shell into the egg.

9. The nuclear cup is left on the outside of the egg; it soon falls off.

10. The wall of the capsule, together with its everted contents, which we now call the sperm-vesicle, sinks into the cytoplasm of the egg, where it is enlarged and transformed into the male pronucleus.

11. The contents of the capsule may be derived from the nucleus of the spermatid and is probably oxychromatin which deposits basichromatin after it enters the egg and so gives rise to the chromosomes in the male pronucleus.

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PLATE 1

EXPLANATION OF THE FIGURES

1 Transverse section of a testicular tubule of a small crab, fixed in Petrunkewitsch's fluid; spermatogonia at the top; spermatozoa at the bottom, spermatids between. × 450.

2 Transverse section of testicular tubule, *p.s.*, primary spermatogonial cell. The mass of spermatogonia larger than in figure 1. × 450.

3 Transverse section of testicular tubule, showing larger mass of spermatogonia; *p.s.*, primary spermatogonial cell. × 450.

4 Transverse section of testicular tubule, showing a large mass of spermatocytes in synapsis; *p.s.*, primary spermatogonial cell. × 450.

5[2] Resting phase of a spermatogonial nucleus.

6 Prophase of a spermatogonial nucleus showing the paired chromosomes which were seen in the upper one-half of the nucleus.

7 Optical section of a spermatogonial nucleus showing the peripheral arrangement of the paired chromosomes in the prophase.

8 The miotic figure in a spermatogonial division.

9[3] Early prophase of the first miotic division of the spermatocyte.

10 Synapsis in the first miotic division of a spermatocyte.

11 to 12 The stage following synapsis, showing the spireme loosened up and separating into chromosomes.

13 First miotic division; stage just preceding the formation of the equatorial plate.

14 Equatorial plate and spindle in first miotic division.

15 A tripolar division of the nucleus of a spermatocyte of the first order. Figure 15a is a drawing of the same nucleus made from a lower plane and showing a portion, *e*, of the chromatin which was not included in the equatorial plate.

16 Chromosomes found in the equatorial plate of the first miotic division.

16 a and b Portions of the first miotic figure in the metaphase, showing the chromosomes.

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[2] Figures 5 to 112 (except 70 to 72) were all drawn with the camera lucida and a Zeiss 1.5 mm. apochromatic objective and a compensating ocular (either a No. 6 or No. 8). Then the drawings were enlarged so that in the plates there is a magnification of 3000 diameters. In making figures 70 to 72 the camera lucida was not used.

[3] Figures 9 to 58 were copied by Mr. E. A. Morrison.

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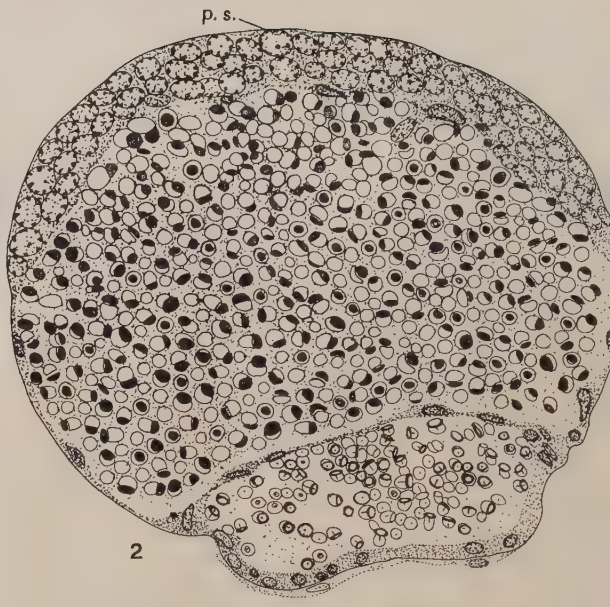

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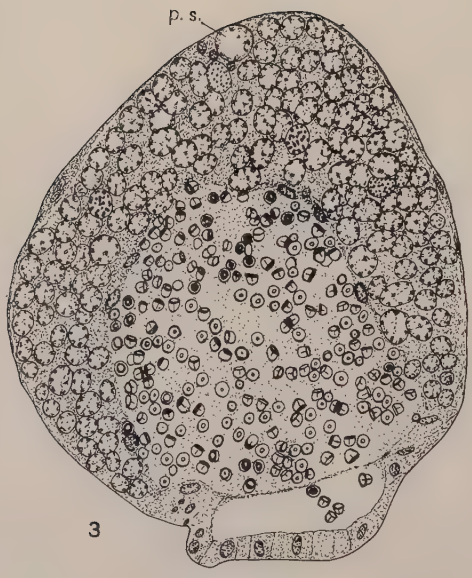

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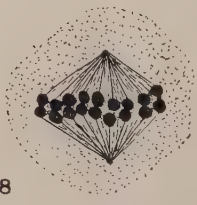

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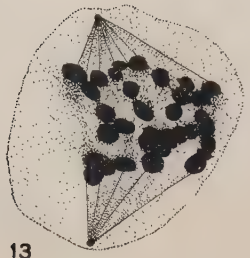

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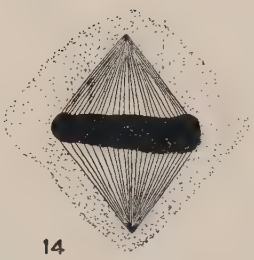

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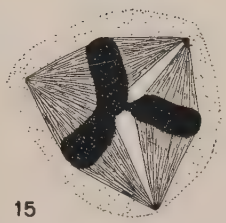

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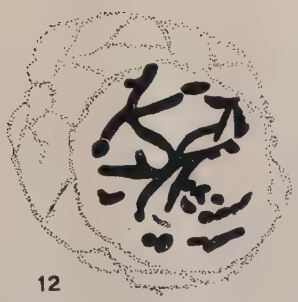

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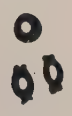

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16a.

16b.

PLATE 2

EXPLANATION OF FIGURES

17 to 18 Two stages in the anaphase of the first miosis.

19 to 22 Show different stages and variations in the early telophase of the first miotic division. Interzonal fibers are shown stretching between the masses of chromatin, the mid-body apparently forming a band around the fibers.

23 to 25 The second miotic division: stages in the formation of the equatorial plate.

26 The metaphase of the second miotic division.

27 Telophase of second mitotic division, showing interzonal fibers and the mid-body.

28 Later telophase. The centrosome is still visible here and is still attached by fibers to the nucleus. A clear area also surrounds the nucleus.

29 to 32 Different stages in the formation of a reticulate nucleus in the spermatid. A black granule, the centrosome may be seen and a more or less complete zone free from granules around the nucleus.

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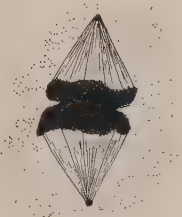
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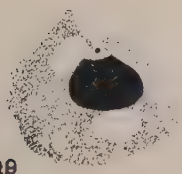
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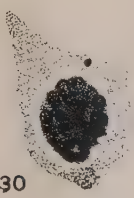
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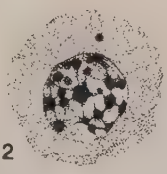
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PLATE 3

EXPLANATION OF FIGURES

33 to 36 Spermatids with vacuoles in the cytoplasm; *c.a.*, mass of finely granular cytoplasm which may be the mitochondria.

37 Spermatid with vacuole or capsule and nucleus; the periphery of nucleus more densely stained than center.

38 Spermatid showing a small clear vacuole in the nucleus.

39 to 42 Later stages in the transformation of the spermatids; *mt.*, mitochondrial substance.

43 Spermatid showing two sides of a dark ring, *d.*, in the capsule; *mt.* mitochondria, also the central body on the nucleus at the bottom of the capsule.

44 Spermatid showing two granules on the border line between the nucleus and capsule.

45 to 47 Spermatid from different view points, showing the mitochondria, *mt.*, and a granule, in bottom of capsule.

48 Spermatid with mitochondrial ring completed.

49 to 60 Spermatids showing stages in the development of the central body and the inner tubule within the capsule. A clear space appears for a time between the capsule and the nucleus; *i.t.*, a vacuole which forms the inner tubule; *c.b.*, central body; *n.*, nucleus.

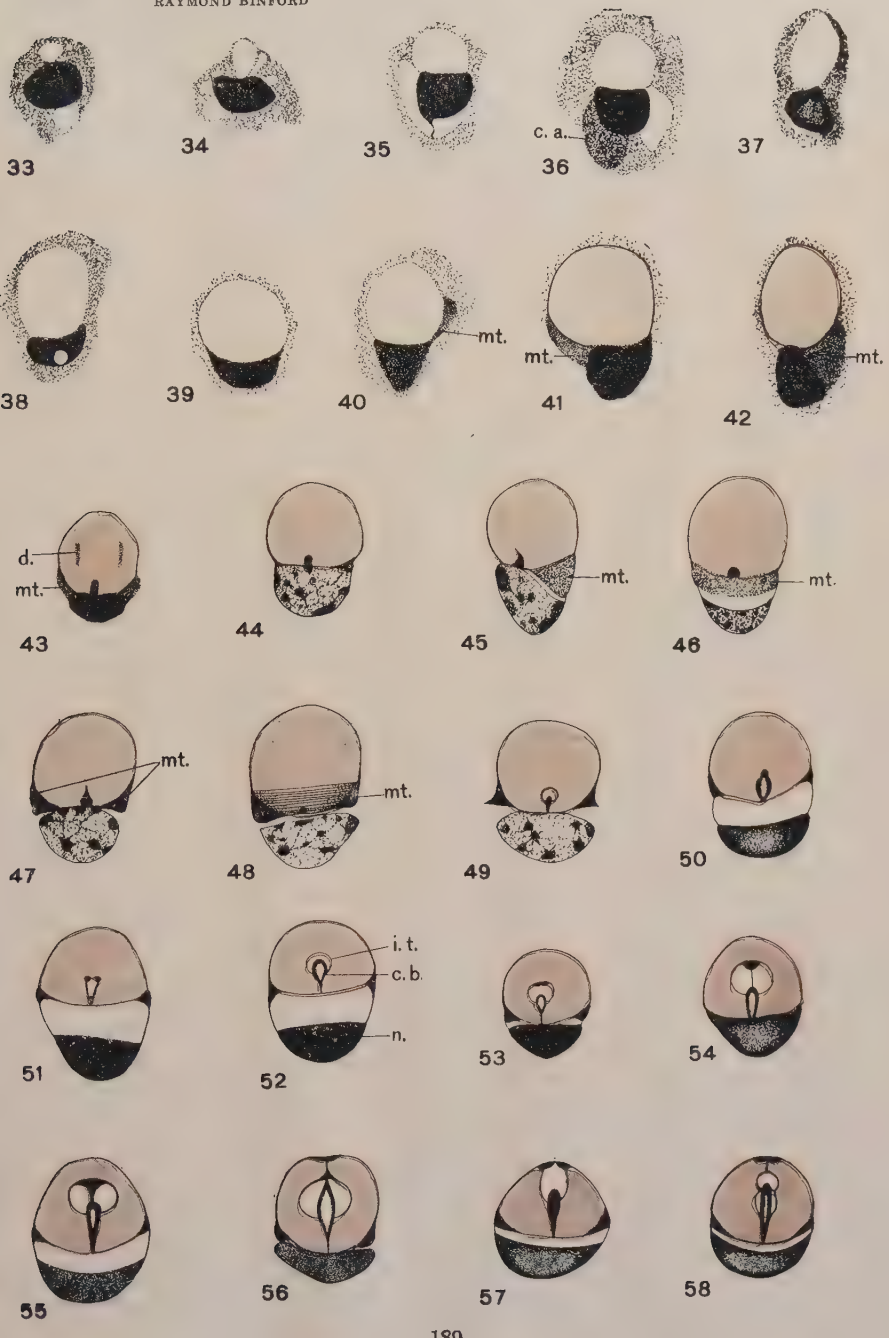
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PLATE 4

EXPLANATION OF FIGURES

61 Mature spermatozoon, fixed in vapor of osmic acid and stained in gold chloride preparation; *n.c.*, nucleus cup; *p.s.*, pseudopodia; *c.*, wall of the capsule; *c.c.*, cavity of the capsule; *i.t.*, inner tubule; *i.t.c.*, inner cavity of the inner tubule, *c.b.*, central body; *o.c.*, outer cavity of the inner tubule.

62 Spermatozoon viewed from the top, mounted in 4 per cent KNO_3.

63 Spermatozoon mounted in the serum of the crab's blood.

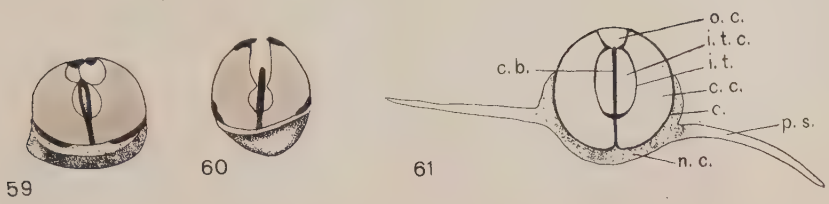
64 Side view of spermatozoon in the serum of the blood.

65 Spermatozoa in serum with pseudopodia all turned to one side by currents in the serum.

66 Spermatozoon in testicular tubule, fixed in Worcester's fluid. Stained in iron-hematoxylin, central body projecting from the top.

67 A spermatozoon treated with 3 per cent KNO_3, fixed with Morgan's fluid, and stained with Delafield's hematoxylin.

68 A spermatozoon treated with 3 per cent KNO_3, fixed with Morgan's fluid and stained with eosin.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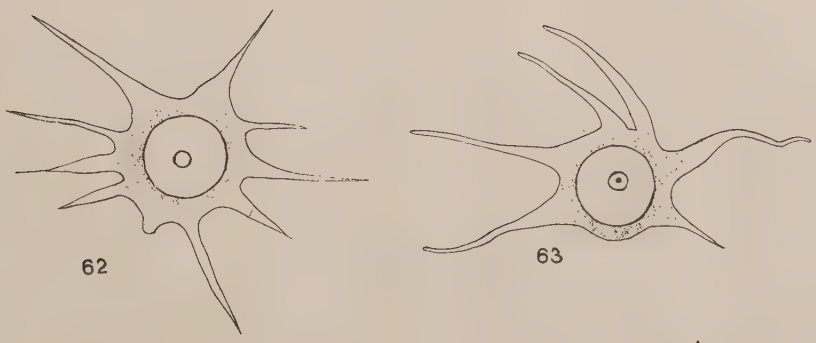
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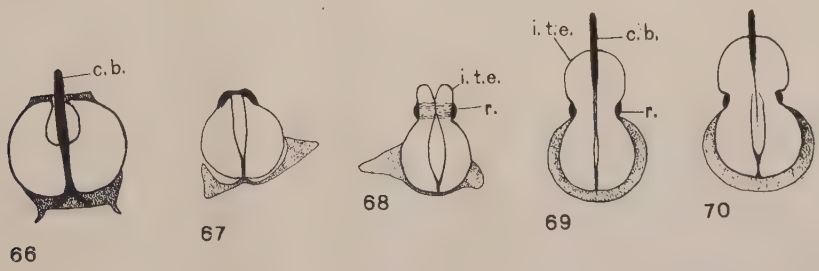
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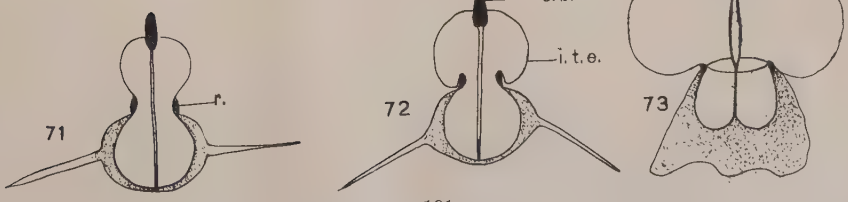
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PLATE 6

EXPLANATION OF FIGURES

93 to 94 Everted spermatozoa in the shells of eggs from the oviduct; *c.b.*, central body ejected; *r.*, collar.

95 The portion of the spermatozoon which remains on the outside of the egg, seen from the bottom of the nuclear cup; *p.s.*, pseudopodia.

96 Spermatozoon on the shell of the egg with nuclear cup next to the shell.

97 A spermatozoon which has exploded, with nucleus next to the egg.

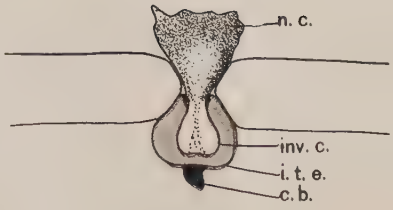
98 to 111 Portions of eggs with spermatozoa, taken from the oviduct or soon after leaving it.

98 to 99 Show the nuclear cup falling away from the egg and pulling a strand of some substance out with it; *n.c.*, nuclear cup.

100 Sperm-vesicle with the strand from which the nuclear cup has broken away, projecting through the shell into the vesicle.

101 Sperm-vesicle just inside the shell.

102 to 103 Sperm-vesicles lying on the cytoplasm.



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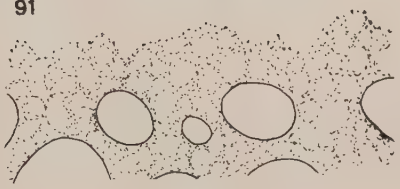

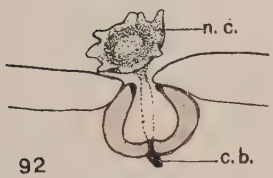

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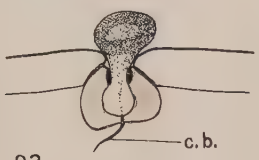

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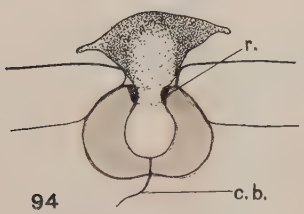

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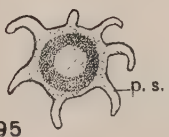

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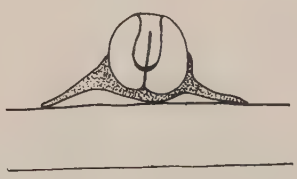

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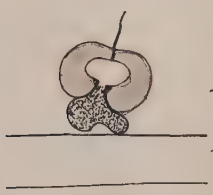

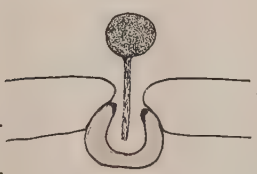

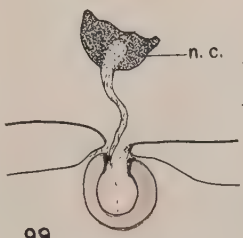

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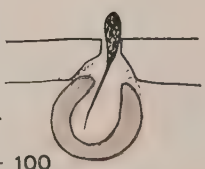

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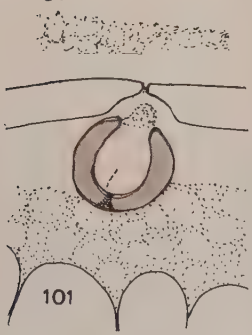

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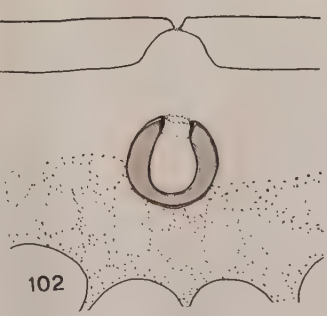

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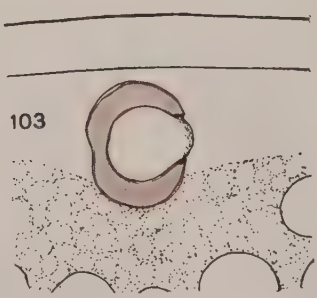
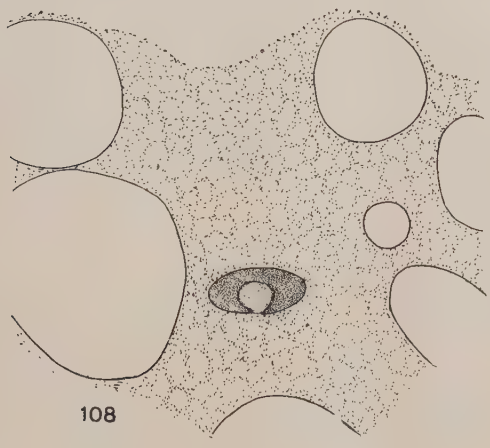


PLATE 8

EXPLANATION OF FIGURES

109 Sperm-vesicle with capsular wall projecting out of it.
110 Sperm-vesicle seen from one side; shows vesicular structure.
111 Sperm-vesicle seen from top—vesicular structure.



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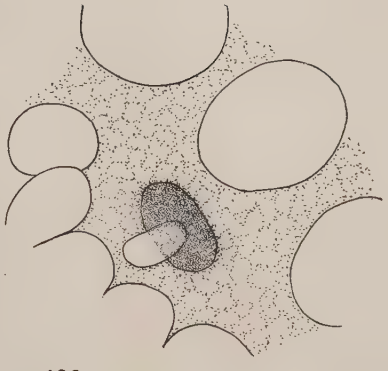

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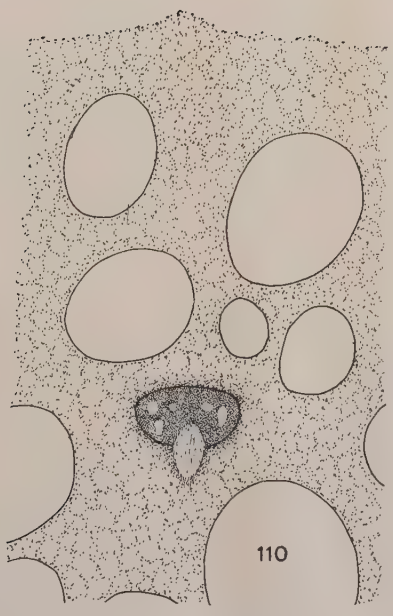

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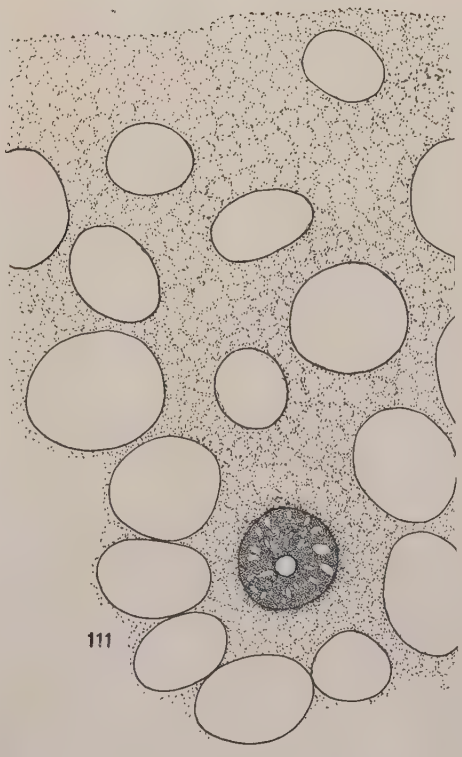

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PLATE 9

EXPLANATION OF FIGURES

112 Sperm-vesicle in egg, one hour and fifteen minutes old. This may now be called the 'male' pronucleus.

113 Egg from the lumen of the ovary showing the spindle of the first miotic division. × 3000.

114 First polar body of an egg just spawned. × 3000.

115 to 116 Two pronuclei found in one egg, two hours and fifteen minutes old. × 3000.

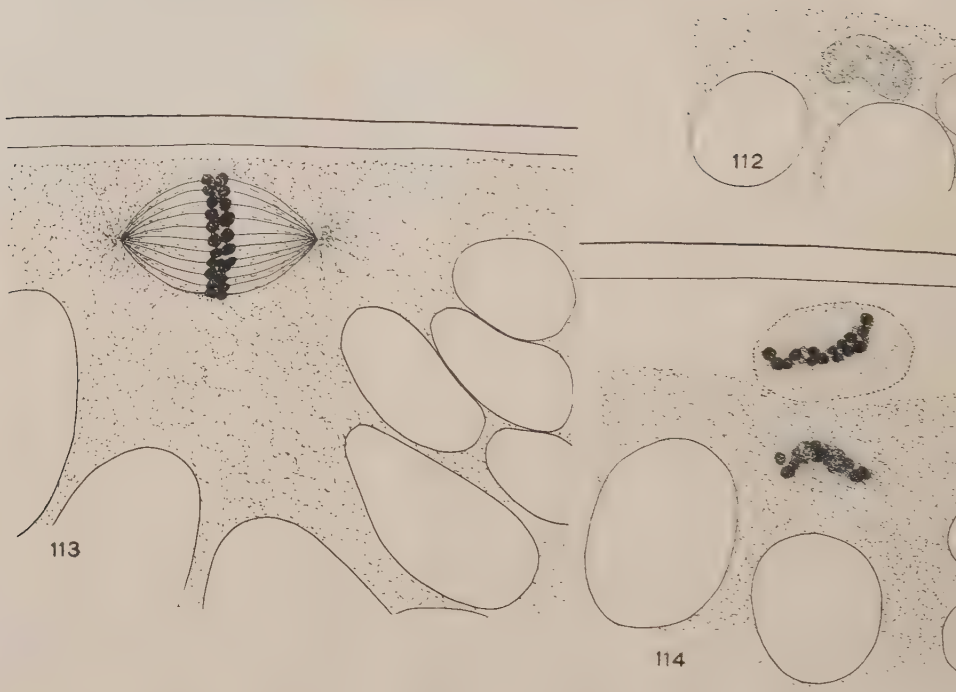
117 Egg two hours and fifteen minutes old; *h.*, transverse section of a hair of the pleopod with the shell of the egg wrapped part of the way around it; *n.*, pronucleus, probably the male; *v.*, vitelline membrane. × 320.

118 to 119 Egg four hours old, showing the pronuclei. Figure 118, × 366; figure 119, × 320.

120 Egg six hours old, showing the pronuclei side by side in the center of the egg. × 320.

121 Diagram of the ovary, the seminal receptacle and oviduct. The latter turned to one side so as to bring it in the same plane with the rest of the ovary; *c.*, portion of the seminal receptacle lined with chitin; *g.*, glandular portion of the seminal receptacle; *o.*, opening between the glandular and chitinous portion of the receptacle; *od.*, oviduct. Natural size.

122 Seminal receptacle at the time of molting; *c.*, chitinous portion; *g.*, glandular portion. Natural size.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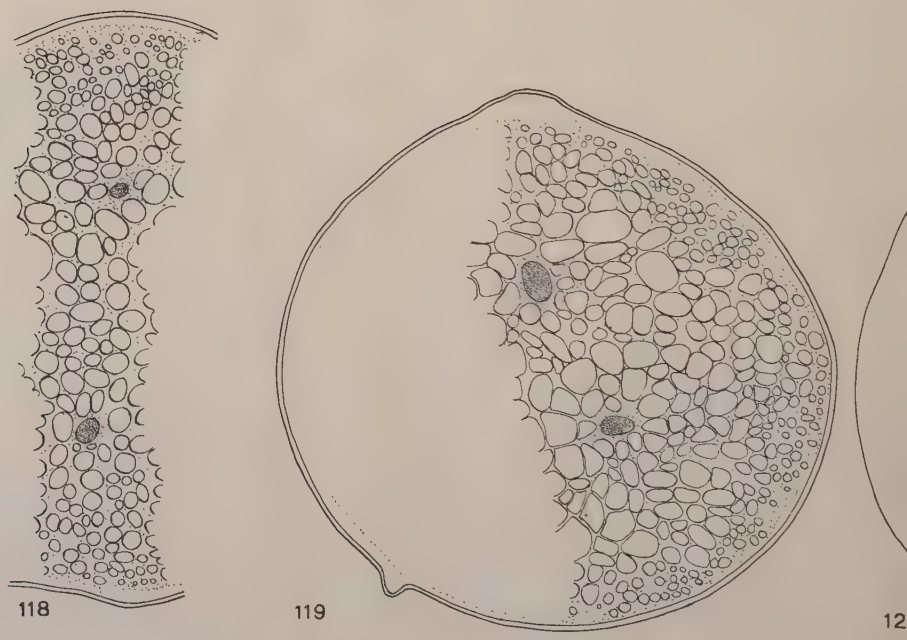
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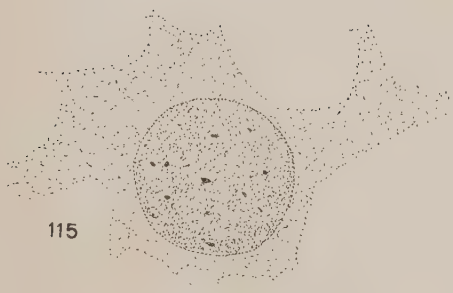

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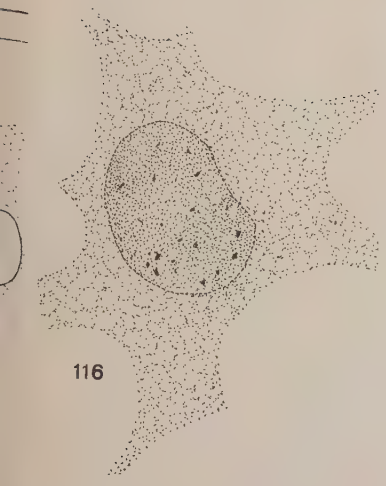

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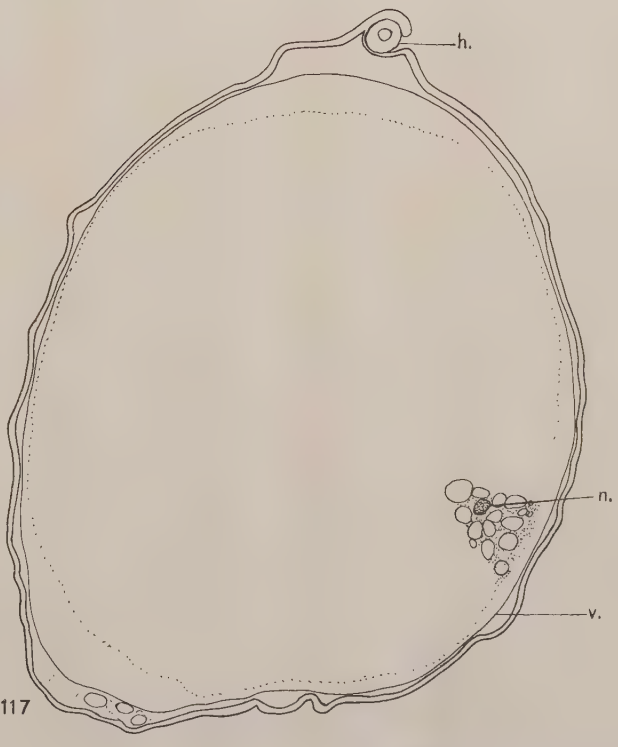

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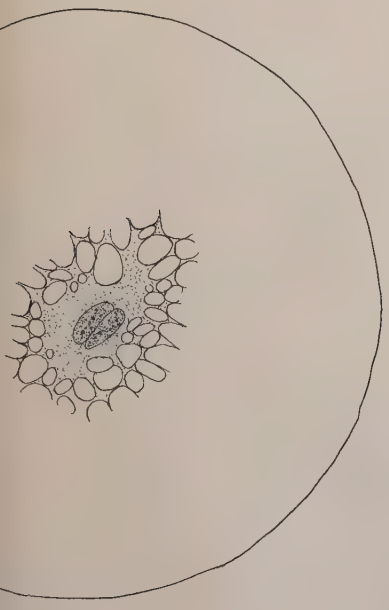

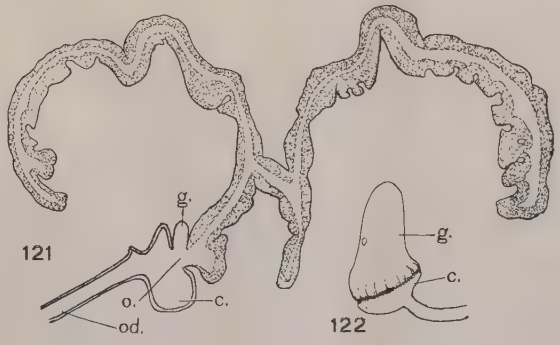

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VITA

I was born near Carthage, Rush County, Indiana, July 15, 1876, and am the son of Josiah and Margaret F. Binford. My family moved to western Kansas in 1885. I graduated from the Haviland Academy in 1896. Then I taught a district school one year, studied at Earlham College, Richmond, Indiana, two years, and again taught a district school at Haviland, Kansas, for one year and the next year returned to Earlham College where I received the degree of B.S. in 1901. Since 1901 I have held the position of Professor of Biology at Guilford College, North Carolina. During the summer quarters of 1902 and 1903, and for five quarters in 1905–1906 I studied in the University of Chicago, devoting my attention principally to the subject of botany, and received the degree of S.M. from that institution.

During the academic year of 1907–1908 I devoted my time to zoological studies at Johns Hopkins University. I was scientific assistant at the United States Marine Biological Laboratory at Beaufort, North Carolina, during the four summers, 1908, 1909, 1910 and 1911. At this laboratory I worked on the life histories of several crabs, though primarily investigating that of Menippe mercenaria.

In the fall of 1910 I returned to the Johns Hopkins University, where the past two years have been spent in working up the foregoing dissertation and in other zoological studies.

RAYMOND BINFORD.

Baltimore, Maryland, May 1, 1912